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Effect of bisphenol-A on neurodevelopment in Drosophila melanogaster larvae

Alexandra M. Streifel
College of Saint Benedict/Saint John's University, amstreifel@csbsju.edu

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Alexandra Streifel

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Approved by:

Jennifer Schaefer

Assistant Professor of Biology

Clark Cotton

Assistant Professor of Biology

Mani Campos

Associate Professor of Biology

William Lamberts

Chair, Department of Biology

Emily Esch

Director, All College Thesis Program

I.   Introduction

Neurodevelopment, or the delicate process of neuronal cell and nervous system development, is an incredibly complex and not fully understood process. Human brain development begins during the third gestational week and continues through adolescence. Neurons form from neural progenitor cells, and the differentiation of these cells is determined by preexisting genetic coding. After differentiation, the neural tube is the first brain structure to be formed. Most of the brain and spinal cord develop from this neural tube, forming a rudimentary Central Nervous System (CNS). Following neural tube formation, neural patterning, the process by which the neural progenitor cells acquire individual identities, begins. Neural patterning closely follows the overall body patterning of the developing embryo. During this patterning step, several functional regions of the CNS further differentiate and develop, including key sensorimotor areas involved in motor function (Stiles & Jernigan, 2010). After neural patterning, neurons themselves develop in respect to the changing structure of the embryo and embryonic nervous system. These neural cells first proliferate and then migrate to the appropriate location in the body, a process mediated by adhesion molecules in the extracellular matrix for neural crest cells or a glial cell called radial glia for neurons of the spinal cord, cerebral cortex, and cerebellum (Purves, Augustine, & Fitzpatrick, 2001). The migrating neurons of the CNS stay within the embryonic neural tube, whereas the neurons of the peripheral nervous system – including both sensory and motor neurons - migrate away from the neural tube and through various other developing body systems before reaching their eventual location in the body (Purves, Augustine, & Fitzpatrick, 2001).

After migration, the neural cell bodies develop dendrites and axons in order to form the extensive information networks of the nervous system. These cells develop a structure called a growth cone that extends toward a target synaptic connection, forming the axon (Stiles &

Jernigan, 2010). Among the plethora of synaptic connections forming during this stage of development is the formation of sensorimotor pathways in the brain, known as the thalamocortical and corticothalamic pathways (Stiles & Jernigan, 2010). This process begins during the second trimester and is completed during the 26th gestational week. This development is crucial in relaying sensory information received at sensors, such as in the skin, retina, cochlea, or from muscle movement, to the thalamus, where this sensory information is integrated and appropriate action is initiated (Stiles & Jernigan, 2010). After birth, this abundance of synaptic connections is pruned in relation to experience and environmental factors. Additionally, oligodendrocytes differentiate and develop, contributing to myelination of neurons; myelination causes a myelin sheath to wrap around the axon of the neuron, thereby insulating the electrical signal generated by action potentials and subsequently speeding up action potential propagation (Stiles & Jernigan, 2010). This process of myelination, synaptic pruning, and some new neuron development continues past infancy, with the prefrontal cortex commencing development in early adulthood.

When these crucial developmental processes in the CNS and PNS are disrupted, neurodevelopmental disorders (NDD) are the result. NDDs encompass a variety of conditions, such as seizure disorders, intellectual disability, and autism spectrum disorders (Maussion, et. al., 2015). However, the exact mechanisms of disrupted neurodevelopment in many NDDs remain a mystery. Abnormal genetic makeup and alterations in gene expression are well-supported factors in NDDs. This variability in gene expression indicates that environmental influences could cause those who are genetically pre-disposed to neurodevelopmental disorders to express the mutations for these disorders or that environmental influences, coupled with genetic markers, account for the variability of NDDs. This new perspective of gene-environment interactions in NDDs is of

particular interest, especially when one takes into account the high levels of industrial chemicals and pesticides that children are exposed to in developed countries from conception – including the most crucial periods of brain development (Szpir, 2006).

Autism is currently a disorder of interest in both scientific research as well as the media. Autism is a disorder defined by abnormalities in three domains: 1. social interaction, 2. language and imaginative play, and 3. range of interests and activities (Muhle, Trentacoste, and Rabin, 2004). Another hallmark of autism and neurodevelopmental disorders is repetitive or stereotyped motor movements, such as spinning in circles or pulling hair repeatedly. According to Muhle, Trentacoste, and Rabin (2004), autism prevalence rates increased by 556% between 1991 and 1997 (Muhle, Trentacoste, & Rabin, 2004). This rise in prevalence rates is largely attributed to greater awareness, stringent pediatric testing for the disorder, and clearer diagnostic criteria, yet some researchers also question whether neurotoxins and environmental factors are potential contributors to the precipitous rise in autism prevalence.

A few chemicals, such as lead or mercury have long been known to cause abnormal development and intellectual functioning (Jiang, Hsi, Fan, & Chien, 2014). Pre-natal or infant lead exposure has been implicated in mental retardation and abnormal development (Jiang, Hsi, Fan, & Chien, 2014). As a result, products such as paint and petrol have been made lead-free for years and mercury regulations have become more stringent so as to decrease the chance of exposure to these chemicals. Now different widely-used chemicals, such as polychlorinated biphenyls (PCBs) and organophosphates (OPs), are of interest in the quest to fight autism, ADHD, and other neurodevelopmental disorders. Jurewicz, Polanska, and Hanke found that, when exposed to organochlorine pesticides, children exhibited deficits in alertness and attention span – even at the miniscule amounts of exposure of 5-10 micrograms per deciliter, which is less

than some environmental exposure levels (Jurewicz, Polanska, & Hanke, 2013). A 2015 study by Lee et. al. found that two industrial pesticides – endosulfan and cypermethrin – caused newborn mice to exhibit altered levels of neuroproteins after a one-time administration of the chemicals. The mice then exhibited altered behavior as adults, such as spontaneous movement and inability to habituate to a new environment, as well as continued decreased levels of neuroproteins, months after exposure (Lee, et. al., 2015). In another study, exposure to phthalates, a common component of plastics that easily leeches into water and the environment, during crucial developmental periods in infancy and childhood resulted in disruption of hippocampal structural and functional plasticity. This disruption may have occurred either as a direct neurotoxic effect of the chemicals or as a result of their role as endocrine disrupting compounds (Holahan & Smith, 2015). In essence, toxic chemicals implicated in NDDs have expanded beyond the common poisons of lead and mercury, yet these new neurotoxins are not heavily regulated like lead and mercury, which may facilitate their contribution to NDDs.

One chemical in particular poses a particularly compelling position in the etiology of disrupted neurodevelopment – bisphenol-A, also known as BPA. This ubiquitous chemical is used in a variety of products, such as the plastics and epoxy resins in containers and water bottles. BPA waste can leach into wastewater and landfills, further impacting both humans and animals (Husain & Quayyum, 2013). One study on BPA revealed that, when injected in rats, the chemical caused an increase in expression of dopamine transporters, resulting in hyperactive behavior characteristic of ADHD and autism. (Brown, 2008). This same study found that BPA exposure also impacted synaptogenesis, apoptosis during neural cell pruning, and myelination of neurons (Brown, 2008). A separate study found that BPA exposure accelerated the neurodevelopmental processes of cell differentiation and migration, resulting in abnormal

cortical patterning and thalamocortical structure, in rats (Nakamura et. al., 2012). BPA also functions as an endocrine disrupting chemical, causing widespread effects on the body. BPA specifically acts as a xenoestrogen, which is a chemical that imitates estrogen, as well as impacts DNA methylation, a process that affects the transcription of DNA (Wolstenholme, Rissman, & Connelly, 2010). Although BPA is man-made, its structure is similar to endogenous estrogen and steroid chemicals, such that it can activate estrogen receptors (Wolstenholme, Rissman, & Connelly, 2010). Besides its neurotoxic effects, the estrogenic factors of BPA result in advanced puberty (Howdeshell et. al., 1999 and Wolstenholme, Rissman, & Connelly, 2010) and decreased testosterone levels (Akingbemi et. al., 2004 and Wolstenholme, Rissman, & Connelly, 2010). BPA exposure caused behavioral changes in studies done on rats, including impaired learning, increased impulsive behavior and decreased interest in novel environments (Wolstenholme, Rissman, & Connelly, 2010). However, the amount of research conducted on the effect of BPA on motor neurons remains sparse.

Despite previous and ongoing studies on BPA in animal and epidemiologic studies on human populations, the mechanisms by which it affects the brain and behavior is still speculative and additional testing is needed to understand how to avoid its toxic effects. Besides the classic experimental animal – the rat – another organism presents a compelling role as a model for neurodevelopmental studies: the fruit fly, *Drosophila melanogaster.*

Drosophila have been used as model organisms in genetics studies as well as neurological research for years. They are useful models due to their large numbers of progeny, short generation time, easily observable behavioral patterns, and simple anatomy. *Drosophila* offer the potential for examination of complex gene-environment interactions that pertain to humans due to the genetic conservation between *Drosophila* and humans (Makay & Anholt, 2006) as well as

the well-developed and widely-available tools for manipulation of the *Drosophila* genome. *Drosophila* also exhibit complex behavioral patterns, such as circadian rhythms, sleep, drug responses, locomotion, and aggressive behavior (Mackay & Anholt, 2006). As a result, *Drosophila* have been used in studies examining many human disorders, such as Parkinson's, Huntington's, alcohol effects, and Alzheimer's. It is logical, then, that *Drosophila* can also function as a useful model in examining neurotoxins and their effect on neurodevelopment, such as BPA.

Drosophila have genetic homology to humans, but obvious differences in body patterns and development. In order to understand *Drosophila* as a model organism for human neurodevelopment, it is important to have a grasp on how *Drosophila* motor neurons – the neuron type explored in this study – develop and how this development differs from or mirrors human neurodevelopment. *Drosophila* neurons develop from neuroblasts, which divide to produce a variety of cells. From 30 neuroblasts, approximately 400 larval neurons are produced, 38 of which are motor neurons (Kim, Wen, & Jan, 2009). The body of the fly larva has 30 muscle segments, which are innervated by one or more of these 38 motor neurons (Kim, Wen, & Jan, 2009). Motor neurons with similar dendrites originate from the same neuroblast and their axons innervate muscles that are functionally related (Landgraf, et. al., 2003). Kim, Wen, & Jan discovered a large degree of overlap between dendritic branches from many neurons, providing full motor coverage and integration through a topographic map of the *Drosophila* body wall (Kim, Wen, & Jan, 2009). There is, however, a gap in understanding how these neurons create specific synaptic connections during development. *Drosophila* neurodevelopment is a complex process liable to disruption leading to adverse effects, much like human neurodevelopment.

Studies on the effect of BPA have been conducted on this model organism in the hopes of investigating the detrimental effects of this chemical on humans and other organisms. *Drosophila* exposed to BPA were more susceptible to starvation due to inhibited lipolysis and inhibited expression of insulin-like peptides (Williams, et. al., 2013). Administration of BPA in both food and through body wall absorption resulted in a marked decrease in fecundity for the flies. This decrease was attributed to the endocrine-disrupting properties of BPA (Atli, 2013). The previous study of BPA and *Drosophila* by Kaur et. al. was the most relevant to this study examined the effects of BPA exposure on behavior in an effort to examine the potential link between BPA and neurodevelopmental disorders. Kaur's study found that adult flies exposed to BPA exhibited greater mobility, angular velocity, turn angle, meander, and number of grooming episodes as compared to control subjects (Kaur et. al., 2015).

The purpose of this study is to examine whether behavioral abnormalities arise in third-instar *Drosophila melanogaster* larvae in response to prenatal BPA exposure, as well as to determine whether morphological differences are caused in peripheral neuronal processes, including motor and/or sensory neurons. Third-instar is the last stage for larvae before they begin to pupate and when larvae begin to "wander" and move around. To date, little is known about morphological differences in neurons of those with NDDs and whether such differences are a response to chemical and endocrine-disrupting compound exposure in neurons. This study expands upon the discoveries of behavioral abnormalities in the Kaur studies by exploring morphological anatomy differences in addition to behavioral changes. Similar methods to the Kaur study were used, but they were adapted to be appropriate to the study of larval behavior as opposed to adult. By administering BPA to the parental generation and testing resultant offspring, this study mimics an environment of prenatal BPA exposure for the etiology of NDDs.

Methods

Fly Stock and Experimental Set-Up

   Drosophila melanogaster from three genetic strains were used: 1010T10, ELAV-GAL4, and UAS-2XeGFP. All fly strains were obtained from a commercial supplier (Bloomington Stock Center). The 1010T10 strain was used for the behavioral assays, and a cross of ELAV-GAL4 X UAS-GFP was used for both behavioral assays and dissection. All fly strains were kept in glass vials in the same room temperature controlled environment under similar light-dark cycles, with light exposure from about 8 AM and 6 PM. All flies were fed a standard diet of Drosophila food (Drosophila Instant Food, Formula 4-24 from Carolina Biological Supply Company, Burlington, NC).

   Drosophila were exposed to one of four conditions: control, 0.44 mM, 4.4 mM, and 44 mM concentrations of bisphenol-A (BPA). BPA solutions were prepared with powdered 97% pure BPA from Sigma-Aldrich (Sigma Aldrich Co., St. Louis, MO). All amounts of BPA were measured using an electronic balance and a plastic weighing boat. The powdered BPA was then transferred to a liter of water and allowed to dissolve. An electronic heating apparatus and magnetic stir bar were utilized to aid in dissolution. The highest concentration of BPA in particular required this equipment in order to dissolve. Once dissolved, the solutions were stored in plastic canisters and kept at 5ºC. The doses represented typical environmental exposure for humans (Atli, 2013). These BPA solutions were used in place of water when preparing the *Drosophila* food.

   All experiments were carried out on F1 third-instar wandering larvae. All experiments were initiated using parental adult *Drosophila* in glass vials filled with prepared *Drosophila* food. During sexing of flies for crosses, adults were anesthetized using carbon dioxide gas and

examined on a Leica EZ4 dissecting microscope (North Central Instruments). Two or three male

flies from the ELAV-GAL4 genetic strain and three to four female flies from the UAS-GFP

genetic strain were identified and transferred to the experimental vial on the head of a paintbrush.

Adult flies were allowed to mate and lay eggs in food made with either water or one of three

BPA doses, and progeny were allowed to mature in the vial to third-instar, approximately a week

after set-up of cross.

Behavioral Assays

All behavioral assays were performed at room temperature and between the hours of 9

AM and 3 PM in order to rule out changes in behavior due to circadian rhythms. Behavioral

assays were based on a study performed by Chauhan et. al. (study cited in article by Kaur et. al.,

2015) and video recorded using a plastic petri dish as an arena for larval movement. The plastic

petri dishes were placed on grid patterned graph paper in order to facilitate quantitation of

locomotor behaviors. A small lamp was shone on the arena in order to aid video analysis. Videos

of larval behavior were recorded using a Canon Vixia Camcorder HF-R Series on a three-leg

tripod (camera rented from Saint John's University Media Services).

Third instar larvae were transferred from vials to the arena using a paint brush. All larvae

were allowed 30 seconds to acclimate to the test environment before videos were recorded. After

the 30 second acclimation period, third instar larvae were recorded for 30 seconds each. Larvae

were either dissected or disposed of after video recording. Video data was analyzed through the

Windows video viewer (videos in MTS format) and the aid of graph paper placed under the petri

dish arenas where the assays were recorded.

Video recordings were analyzed for four behavioral paradigms commonly exhibited by

third instar *Drosophila* larvae: time spent in seeking behavior, number of peristaltic contractions,

linear movement, and angular movement. All video analyses were conducted using careful naked eye analysis of each video. In order to provide the most accurate analysis possible, operational definitions of each behavioral paradigm were defined prior to analysis. Definitions were based on Kaur, et. al. and for larvae. *Time spent in seeking behavior* was defined as the number of seconds the larvae spent stationary moving their heads repeatedly and in an exploratory fashion. *Number of peristaltic contractions* were defined as the number of times in a 30 second period that a wave of peristaltic muscle contraction moved from the head of the larvae to the tail, or vice versa. *Linear movement* was defined as the linear distance a larvae traveled within the 30 second recording period. Linear movement was measured using the underlying graph paper and was recorded in respect to the number of graph squares the larvae moved. *Angular movement* was defined as the number of times the larvae changed direction within the 30 second period and an estimation of the degree of each change from initial body position. These recordings were also made with respect to the underlying graph paper squares. Twenty-five replicates of each condition were analyzed, with 25 organisms in each condition tested for all paradigms. The mean, standard error, and one-way ANOVA of each condition were recorded from this data. ANOVA was followed by t-test with Bonferroni correction (p-value).

Dissection and Motor Nerve Morphological Analysis

Five larvae from each condition were dissected for morphological analysis. Larvae were transferred from the plastic arena used for behavioral assays to the agar-coated petri dish used for dissection via the end of a paintbrush. The dissection dish was then placed under a dissection microscope (Leica EZ4, North Central Instruments). Larvae were pinned, ventral side down, to agar in dish using insect pins and forceps. Pins were placed in tail and close behind mouth combs at head of larvae. The dish was then filled with PBS saline. The PBS solution was prepared with

130 mM NaCl, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4 X H_2O, and water until the solution was a liter in volume. The body wall of the larva was cut longitudinally along the dorsal midline between paired trachea using a microscissors and pinned open in a fillet preparation. All organs were removed from the animal with the exception of the brain nerve cord, and peripheral nerves. When the animal was cleanly dissected, leaving the peripheral nerves intact, the PBS was discarded and the specimen was prepared for the staining procedure.

The dissected specimen was fixed and stained using immunohistochemical staining. The dissected larvae were fixed in 4% paraformaldehyde at room temperature for 30 minutes. The specimen was then washed three times for ten minutes each in TBS, which was produced by adding Triton X to prepared PBS in a 1:100 ratio. After the wash, the specimen was blocked and agitated for 30 minutes. The block solution used was comprised of 10 mL of normal goat serum (NGS), 4 g of bovine serum albumin (BSA), 20 mL TBS, and water to reach a volume of 200 mL. At the conclusion of the block period, primary antibody, anti-GFP, was applied to the preparation in a 1:1000 ratio in block and incubated overnight in a refrigerator. After the incubation period, the specimen was washed and agitated for three ten-minute washes in block solution. The secondary antibody, Alexa 488, was added to the block in a 1:500 ratio and incubated for 1-2 hours a room temperature. The specimen was washed 3 times for 10 minutes in TBS and once for 10 minutes in PBS before being unpinned and mounted on a glass slide.

In order to visualize the stained specimens, the prepared slides were viewed using a fluorescent microscope (Olympus IX71) using a blue excitation wavelength. Fluorescence images and brightfield images were taken at 10x, 20x, and 40x magnifications. The neuromuscular junction between muscles 6 and 7 were examined for qualitative appearance of axon terminals, and significant differences in the surface areas of terminal boutons. The mean,

standard error of the mean, one-way ANOVA, and t-tests with Bonferroni correction were collected from these analyses.

II. Results

<u>Behavioral Assays:</u>

Four conditions of larvae were studied in this experiment: control, .44 mM BPA (A), 4.4 mM BPA (B), and 44 mM BPA (C) experimental conditions. Larval behavioral data was analyzed in regards to five behavioral paradigms: time spent in seeking behavior, number of peristaltic contractions, linear movement, angular movement, and degree of turns. Easily observable qualitative differences in larval behavior were also noted and will be detailed below.

Time Spent in Seeking Behavior

Time spent in seeking behavior was assessed as the number of seconds that an individual larva spent exploring its environment within a 30 second testing window. The data collected from this behavioral assay is shown in Figure 1. The control group spent an average of 8.12 seconds engaged in seeking behavior. The experimental groups A-C showed increased time spent in seeking behavior, with Group A spending an average of 9.24 seconds, Group B spending 11.24 seconds, and Group C spending 15.28 seconds in seeking behavior within a 30 second testing window. Group C, with a 44 mM concentration of exposure of BPA was the only experimental group that varied significantly from control. From a one-way ANOVA analysis, the F ratio for seeking behavior was over 1.0, with a value of 4.5. The ANOVA analysis also showed a low P value, of 0.0053. The best test of significance was t-tests among the various experimental groups. Due to multiple comparisons among data, a Bonferroni correction was run, which indicated a significance value of 0.0167. P-values comparing each BPA treatment group

to the control are as follows: .44 mM (0.59), 4.4 mM (0.17), and 44 mM (0.006). Since the 44

mM condition showed a t-test p-value, it is statistically significant.

Number of Peristaltic Contractions

Number of peristaltic contractions was assessed as the number of times in a 30 second

testing window that a wave of peristalsis moved from tail to head or vice versa. The control

group exhibited a mean value of 6.64 contractions per 30 second testing window. The three

experimental groups all showed significantly increased numbers of peristaltic contractions, with

Experimental Condition A undergoing an average of 9.56 contractions, Experimental Condition

B undergoing 10.6 contractions, and Experimental Condition C undergoing 12.36 contractions,

all within a 30 second testing window (Figure 2). From one-way ANOVA analysis, the F ratio

was higher than 1.0, with a value of 8.69, and the P value was 3.72×10^{-5}. Due to the unequal

variance among all conditions, a Bonferroni correction was run on the data, with a new

significance threshold calculated to be 0.0167. The t-test p-values comparing each BPA

treatment to the control were as follows: .44 mM (0.003), 4.4 mM (0.0001), and 44 mM (3.25 X

10-5). All conditions had p-values lower than this value, indicating statistical significance.

Linear Movement

Linear movement of each larva in a 30 second testing window was measured as the

number of graph paper squares that each larva traversed in the testing window. The number of

squares was measured using the graph paper placed under each clear petri dish arena. To

distinguish this data from angular movement, the larva must move along one of the lines on the

graph paper or without diverting its body orientation from initial body positioning. The control

group moved an average of 0.02 graph paper squares in a 30 second testing window. The three

experimental groups exhibited significant increases in the number of graph paper squares moved

within a 30 second testing window, with Experimental Condition A traversing 0.11 squares, Experimental Condition B traversing 0.26 squares, and Experimental Condition C traversing 0.38 squares per 30 seconds (Figure 3). From one-way ANOVA analysis, the F ratio was calculated to be greater than 1.0, at a value of 5, and the P value was calculated to be 0.0029. The p-values comparing each BPA treatment to the control were as follows: .44 mM (0.0029), 4.4 mM (0.003), and 44 mM (0.003). Due to unequal variance among conditions, a Bonferroni correction was run on the data, with a corrected significance threshold of 0.0167. All conditions had t-test p-values lower than this corrected significance threshold.

Angular Movement

Angular movement for each larva was recorded as the number of times that each larva changed direction from initial orientation in a 30 second testing window. The control group turned an average of 1.48 times within the 30 second testing window. Experimental Condition A turned an average of 1.56 times, Experimental Condition B an average of 2.76 times, and Experimental Condition C an average of 2.84 times (Figure 4). From one-way ANOVA analysis, an F ratio was calculated over 1.0, with a value of 3.5, and a P value was calculated to be 0.012. The t-test p-values comparing each BPA treatment to control were as follows: .44 mM (0.89), 4.4 mM (0.03), and 44 mM (0.04). Due to unequal variance among all conditions, a Bonferroni correction was run, which resulted in a corrected significance threshold of 0.0167. With the standard significance threshold of 0.05, the 4.4 mM and 44 mM conditions would have been statistically significant, but with the corrected value, none of the conditions showed statistically significant differences from the mean.

Degree of Turn

Degree of turn estimated as the degrees of each angular turn that each larva completed during a 30 second testing window. These turn degree values were estimated using initial body positioning as the point of reference and the graph paper underlying the clear petri dish arena as a helpful guide. Due to the varying numbers of turns within each group, the population sizes are not equal across the four groups. The control condition exhibited an average turn degree of 41.51 degrees. Experimental Conditions A and B decreased in turn angle value from control and Experimental Condition C increased in turn angle value from control. Experimental Condition A showed an average turn angle of 28.26 degrees, Experimental condition B an angle of 29.79 degrees, and Experimental Condition C an angle of 53.29 degrees (Figure 5). From a one-way ANOVA analysis, an F ratio was calculated over 1.0, with a value of 6.5, and a P value was calculated to be 0.0003. The t-test values are as follows: .44 mM (0.096), 4.4 mM (0.079), and 44 mM (0.13). Due to unequal variance among the conditions, a Bonferroni correction was run, resulting in a corrected significance threshold of 0.0167. All conditions did not meet this threshold and did not exhibit statistical significance.

Qualitative Observations

A few important qualitative observations were notated during the behavioral assays. For the control group, one larva came into contact with dried Drosophila food, potentially impacting its locomotion in the sticky dried substance. Another larva was markedly smaller than the other tested larvae, suggesting either small-for-development size or a younger than third-instar larva was tested.

For the .44 mM (A) group, one larva rolled over three times during the 30 second testing window, and one larva rolled over once during the recording. One larva lifted its body up once during the testing window, and one larva came into contact with a dried Drosophila food spot,

such as in the control condition. Two larvae had their rear or tail ends elevated during most of the 30 second recording.

For the 4.4 mM (B) group, three larvae had their rear or tail ends elevated during most of the recording window. One larva exhibited notable lengthwise stretching during most of the recording, and one larva moved its tail end considerably during most of the 30 second window.

For the 44 mM (C) group, the tested larvae were markedly smaller than the other conditions, indicating developmental abnormalities resulting in small-for-development sizes. One larva came into contact with a dried Drosophila food spot, much like the control and .44 mM conditions. Two larvae had their rear or tail end elevated for one-third of the testing window, whereas another larvae exhibited this behavior for approximately one-half of the recording.

Neuron Morphology

Synaptic Area

Five specimens each of the control, .44 mM (A), and 4.4 mM (B) conditions were dissected, stained immunohistochemically, and mounted on slides. Pictures of these slides were taken on a fluorescent microscope under blue and white light. Due to challenges with mold and insufficient staining, images of GFP and brightfield were collected only for the control and 4.4 mM BPA (B) conditions. Qualitative abnormalities in neuron morphology were also be noted in slides. The 4.4 mM condition was selected to be the most useful dosage in determining the effect of BPA on neuron development and morphology due to the fact that lower dosages might not show any changes and the highest dosage was potentially lethal, often resulting in the deaths of the adult flies placed in these high dosage vials prior to breeding.

The area of muscles 6 and 7, the length of the neuromuscular junction innervating this muscle, and the ratio of length:area were measured in the resulting slides. For the control flies (c360), the area was found to be 2.27 X 10e5 units on imageJ with a standard deviation of 2.4 X 10e4, a neuromuscular junction length of 128 units and standard deviation of 1.4, and a length:area ratio of 5.63 X 10e-4 and a standard deviation of 5.35 X 10e-5. For the experimental condition (4.4 mM, or B, in dish 36444), the area was 2.09 X 10e5 units with a standard deviation of 6.48 X 10e4, a neuromuscular junction length of 208 units with a standard deviation of 33.42, and ratios of 9.94 X 10e-4 with a standard deviation of 2.19 X 10e-4.

Due to the shortage of data, it is difficult or near impossible to calculate averages from populations of one or two data points. To compare these values to the control condition, the area of the control condition area was 18,077.25 units greater than the experimental condition. The neuromuscular junction for the control condition had a neuromuscular length 80 units less than the experimental condition, which is multiple standard deviations from the mean.

In regards to the neuromuscular length to muscular area ratio, the control ratio was 4.31 X 10e-4 units less than the experimental condition, which is a few standard deviations away from the mean.

Qualitative Observations

As stated above, higher dosages of BPA often resulted in a higher rate of adult fly deaths prior to breeding. Higher dosages of BPA often appeared to be more susceptible to mold than control. Additionally, higher concentrations of BPA often resulted in lower fecundity and smaller larval size than control. It was also observed that the time between the genetic crosses being set up and the development of third instar progeny was greater for the experimental conditions with higher BPA dosages and this gap was largest for the 44 mM (C) experimental condition.

Fluorescent images of the CNS for each condition were taken and analyzed for qualitative changes. From a qualitative analysis, the CNS of the control (c360) condition had fewer nerves emanating from the CNS than the experimental (36444) condition.

III.   Discussion

Behavioral Assays

The potential effect of bisphenol-A on *Drosophila melanogaster* neurodevelopment was explored using five behavioral paradigms. These behaviors were chosen due to three factors: 1.) they are common larval behaviors that would be manageable to measure using video analysis, 2.) they are similar to and therefore allow comparisons to behaviors measured by Kaur, et. al in adult Drosophila, and 3.) Drosophila behaviors correlate to common behavioral abnormalities caused by neurodevelopmental disorders. It is recommended that, in future experiments, analytical software be used to better quantitatively assess Drosophila behavior during behavioral assays.

Neurodevelopmental disorders are characterized by hypersensitivity to sensory stimuli and a variety of behavioral abnormalities, including those of stereotyped and repetitive behaviors (Futoo et. al., 2014). The Kaur study demonstrated that Drosophila behavior provides a quantifiable model for BPA-mediated neurodevelopmental abnormalities in their finding that adult fly social interaction was abnormal in BPA treated flies, mimicking unusual social behavior exhibited in neurodevelopmental disorders such as autism. In general, behavioral changes in response to administration of a suspected neurotoxin offer the opportunity to explore the effects of this chemical on disorder etiology and may point toward a stronger than correlational relationship between exposure to substances such as BPA and neurodevelopment.

Seeking, a common larval behavior that is not observed in adult flies due to differences in body shape and structure, is a correlate to altered sensory sensitivity characteristic of autism and other disorders of neurodevelopment. During seeking behavior, third-instar larvae use sensory structures located near their mouth combs and head end to explore their sensory environment as well as to direct movement. Only the highest dosage group of larvae showed a statistically significant increase in time spent in seeking behavior, this change in behavior can only be definitively attributed to high doses of BPA exposure. It is important to note that recordings were conducted in an open arena and in the presence of approximately two other larvae. The element of the open field assay could affect seeking behavior. Open field assays are often used as measures of anxiety in animal research. Within a petri dish arena, effects due to anxiety of a new environment were minimized due to a 30 second acclimation period prior to recording. However, larvae may still have been acclimating to the environmental change during the testing window, thereby affecting seeking behavior as they explore their new, open environment. Nonetheless, there is no reason to believe that the effect of this environment would be different between the control and treatment groups.

Peristaltic may be analogous to repetitive and stereotyped motor behaviors that are a hallmark of some neurodevelopmental disorders. Since all experimental conditions exhibited a statistically significant increase in peristaltic contractions from control, it can be asserted that exposure to BPA causes an increase in repetitive behavior in Drosophila melanogaster. The number of peristaltic contractions, on average, also increased with increased BPA dosage. This increase may indicate that prenatal BPA exposure causes an increase in repetitive or stereotyped movements, with severity increasing with increasing BPA exposure concentration.

Linear movement distance provided a measure of how far each larval group traveled and moved around their environment within a 30 second testing window. Greater linear movement may indicate greater sensory exploration and/or differences in circuitry. Since each experimental condition exhibited a significant increase in the number of graph paper squares traversed in a linear fashion within the 30 second testing window, it can be asserted that exposure to BPA results in increased linear movement and sensory exploration.

Number and degree of angular turns provided a measure of sensory exploration and locomotion, similarly to linear movement. Interpreted together, angular and linear movements give a measure of how much each larval condition moved about the petri dish arena in the given 30 second testing window. Since none of the groups exhibited either statistically significant differences in number of angular turns or degree of angular turns, the null hypothesis would fail to be rejected and all differences would be due to chance. This is an important finding that coincides with the statistically significant linear movement data as well as the seeking behavior data, both of which pertain to exploratory sensory behavior. This failure to reject the null could indicate that abnormalities in sensory exploration and movement due to BPA exposure in Drosophila are either smaller in magnitude than that seen in the linear movement and seeking behavior paradigms, or that any differences in sensory exploratory behavior in response to BPA exposure occur in some behavioral patterns but not all. This could indicate a greater potency of BPA in the development of certain neuron types or locations within the CNS or PNS.

Morphological Anatomy

Despite the shortage of concrete data from slides, it was possible to make conjectures from this sparse data as well as published studies. According to a 2015 study by Kimura, et. al., mice exposed to BPA during development were tested to determine the effect of BPA exposure

on neurons in the hippocampal area. Kimura, et. al. discovered that BPA exposure resulted in decreased length and number of branches for basal dendrites in the hippocampal neurons (Kimura, et. al., 2015). Additionally, a study by Ling, et. al. found that neuronal migration in the central nervous system was disrupted in a dose-dependent manner (Ling, et. al., 2016). These studies help facilitate the interpretation of the results of this study

Previous studies, such as those by Kimura et. al. and Ling et. al., have discovered that prenatal BPA exposure in rats results in a decrease in dendritic length and branching density. In other words, BPA in certain doses results in either decreased development of these receptive or synaptic structures or increased pruning of dendrites. Based on the finding that the length of the dendrites was diminished, one could predict that the prevailing effect of prenatal BPA exposure is halted development of these dendritic structures. With this assumption, the likely mechanism of BPA action on the development of dendrites is through decreasing the amount of synaptogenesis during neurodevelopment and diminishing the amount of signaling molecules involved in the creation of these synapses. BPA exposure may also impede the growth of these dendrites and prematurely stunt their lengthwise growth. The net result of this decreasing dendritic surface area would presumably be less communication between neurons and fewer connections between them, diminishing the coordination and initiation of certain nervous functions. From a basic standpoint, this could be linked to a lack of control over stereotyped movements and decreased development of social function, characteristic of NDDs. However, according to a 2014 study by the University of North Carolina School of Medicine, genetic evidence shows that increased dendritic growth, rather than decreased, may be an autism risk factor, further complicating the relationship between behavioral symptoms and neuronal development.

These conflicting studies complicate the interpretation of the effect that BPA in particular has on neurodevelopment and what behavioral changes occur as a result of these effects. However, it is important to distinguish between the specifics of these studies to best come to conclusions on the effect of BPA on neuron morphology, if any. In the Kimura et. al. study, for example, the diminished dendritic branching and dendrite length was detected specifically in the hippocampus. This study focused motor neurons, specifically those involved in innervating muscles 6 and 7 of the *Drosophila* body wall. While it is plausible that chemical exposure has the same effect on all neurons, make conjectures about the expected outcome of this study based on the Kimura et. al. study may be inaccurate. Perhaps the greatest inaccuracy in using this study as a way to make predictions on BPA's effect on motor nerves is the behavioral effects that would stem from both outcomes. Diminished neuron-to-muscle surface area ratios in the hippocampus would have greatly different effects on behavior and motor function – which were studied in the other part of this study – than would this ratio in motor neurons, which were the particular targets in this study. In order to make predictions on a potential decrease in dendrite surface area on the motor neurons specifically, one would have to differentiate any hippocampus-specific effects from the Kimura et. al. study from those that could be expected for other types of neurons.

The most important values in determining the effect of BPA on motor neuron development is by examining the neuromuscular junction length to muscle area ratios. It is difficult to compare since the ratios for the experimental GFP and brightfield junctions have standard deviations of 0. However, the experimental condition has larger length-to-area ratios than control conditions. This aligns more with the University of North Carolina School of Medicine findings if one interprets these results to mean that BPA exposure results in greater risk

for NDDs. In other words, BPA exposure causes an increase in dendritic length along the neuromuscular junction in comparison to control, yet due to the extremely small data pool, these results cannot be declared statistically significant. Longer dendrites along neuromuscular junctions could result in greater activation and firing of these muscles, resulting in the increased motor activity, such as seeking behavior, peristaltic contractions and linear movement, discovered in the behavioral analyses. The qualitative observation of the control CNS showing fewer nerves emanating from the brainstem than experimental also aligns with this discovery.

IV.   Conclusion

In conclusion, the administration of BPA in the prenatal environment had significant impacts on some aspects of Drosophila behavior, as well as potentially significant impacts on motor nerve morphology. These findings are an interesting look into the way a ubiquitous neurotoxin acts upon the delicate process of neurodevelopment. The conclusions reached by this study support previous findings of BPAs effect on both behavior and anatomy in the developing organism as well as opens discussion as to future directions in researching this chemical.

V.   Acknowledgements

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VI.   References

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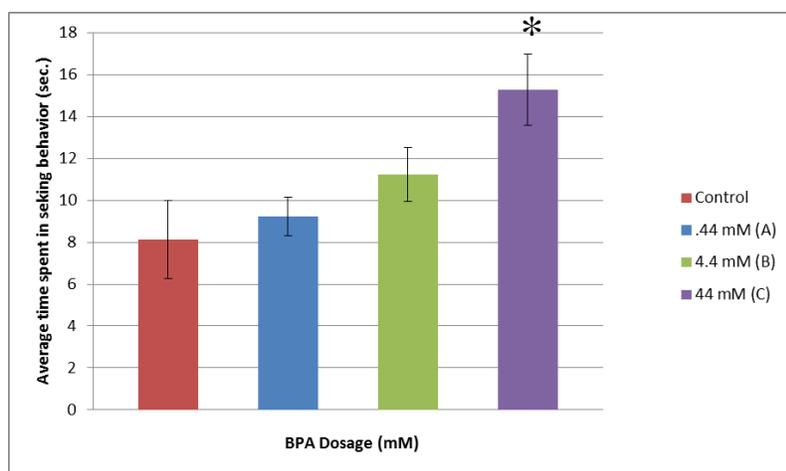
VII.   Supplemental Materials

Figure 1: Mean time spent in seeking behavior(in sec.) for control and three varying levels of BPA dosage (in mMol) for third instar *Drosophila melanogaster* larvae. Mean values for the four conditions are as follows: control (8.12 sec.), .44 mM (9.24 sec.), 4.4 mM (11.24 sec.), and 44 mM (15.28 sec.). Standard error, as shown by the error bars present on the bar chart, was as follows for each condition: control (1.85), .44 mM (0.91), 4.4 mM (1.28), 44 mM (1.69). After t test analysis, the .44 and 4.4 conditions were not statistically significant from the mean (0.59 and 0.17, respectively). The 44 mM condition had a t-test value of 0.006, lower than the null hypothesis threshold of 0.05. Due to unequal variance, a Bonferroni correction was run, setting the significance threshold at 0.0167, which the 44 mM still meets, further proving its significance. The F ratio for this test was 4.5 and the P value from ANOVA analysis was 0.005.
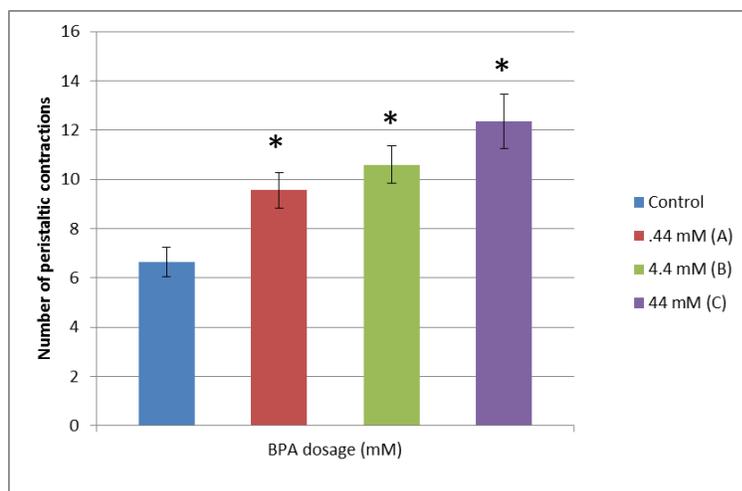

Figure 2: Number of peristaltic contractions observed within a 30 second testing window for varying dosages of BPA (mM) in third instar *Drosophila melanogaster* larvae. Mean values for each condtion are as follows: control (6.64 contractions), .44 mM (9.56 contractions), 4.4 mM (10.6 contractions), and 44 mM (12.36 contractions). The standard error for each condition, as shown graphically by the error bars for each column, were as follows: control (0.59), .44 mM (0.73), 4.4 mM (0.75), and 44 mM (1.1). After t-test analysis, all conditions were found to be statistically significant from the mean, with values as follows: .44 mM (0.0031), 4.4 mM (0.00013), and 44 mM (3.25E-05). All values are lower than the significance threshold of 0.05. Due to unequal variance among the various condtions, a Bonferroni correction was run, setting a corrected significance threshold of 0.0167 for the data. All conditions are lower than this value, further proving that any differences are not due to chance. The F ratio from one-way ANOVA analysis was calculated to be 8.69 and a P value was calculated to be 3.72E-05.
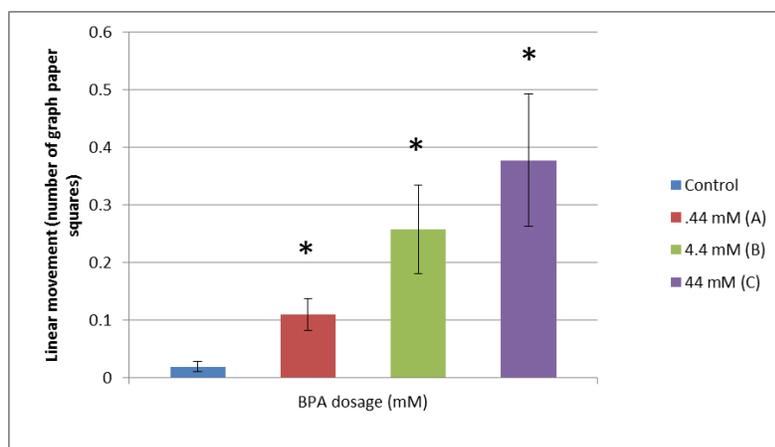
Figure 3: Linear movement (in number/fraction of graph paper squares) observed in a 30 second testing window for third instar *Drosophila melanogaster* larvae of varying BPA dosage conditions. The mean values for each condition were as follows: control (0.02 squares), .44 mM (0.11 squares), 4.4 mM (0.26 squares), and 44 mM (0.38 squares). Each group exhibited variation in linear movement, as shown graphically by the error bars. The standard error values for each condition were as follows: control (0.009), .44 mM (0.027), 4.4 mM (0.076), and 44 mM (0.115). All values showed statistically significant means from control, according to the commonly accepted 0.05 value. The t-test values for each condition were as follows: .44 mM (0.0029), 4.4 mM (0.003), and 44 mM (0.003). Due to unequal variance among each condition, a Bonferroni correction was run on the data, yielding a corrected significance threshold of 0.0167. After this correction, all conditions were still found to be statistically significant. From one-way ANOVA analysis, an F-ratio was calculated to be 5, and a P value was calculated as 0.0029.
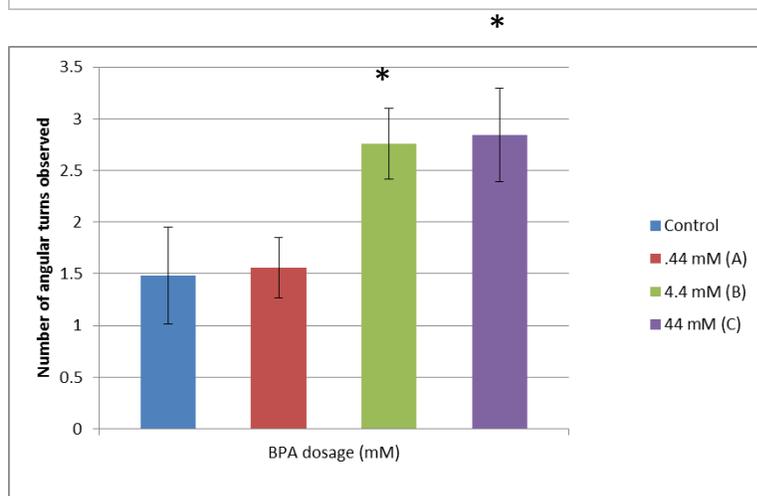

Figure 4: Number of angular turns as observed in a 30 second testing window of third instar *Drosophila melanogaster* larvae of varying BPA dosing conditions (in mM). The mean values for each condition were as follows: control (1.48 turns), .44 mM (1.56 turns), 4.4 mM (2.76 turns), and 44 mM (2.84 turns). Each condition exhibited variability, as shown by the error bars on each column. The standard error for each condtion were as follows: control (0.47), .44 mM (0.29), 4.4 mM (0.34), and 44 mM (0.45). The t-test values for each condition were calculated to be .44 mM (0.89), 4.4 mM (0.032), and 44 mM (0.043). The 4.4 and 44 mM conditions were found to be statistically significant from the mean accoridng to the commonly accepted significance value of 0.05. Due to the unqual variance among all conditions, a Bonferroni correction was run on the data and yielded a corrected significance value of 0.0167. After this correction, none of th conditions were statistically significant from control. From one-way ANOVA analysis, a F ratio was calculated as 3.5, and a P value was calculated at 0.019.
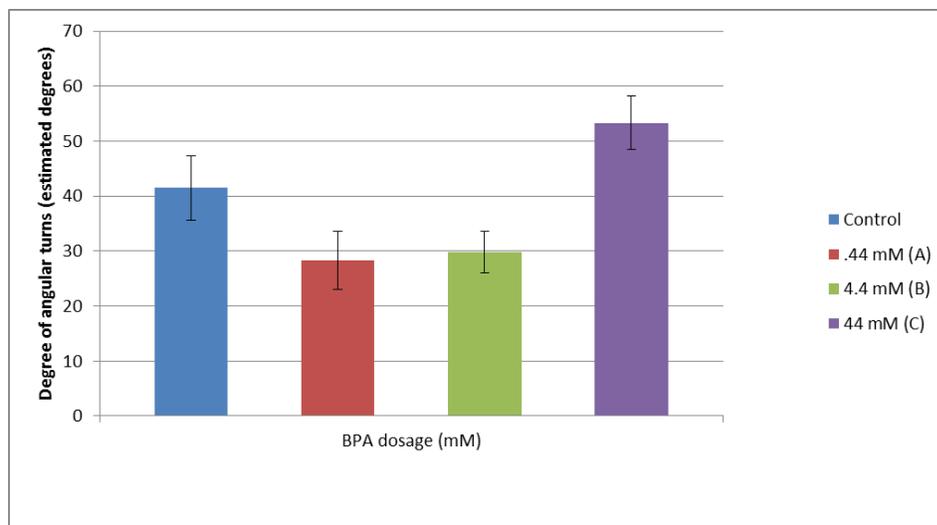
Figure 5: Estimated degree of angular turns (in estimated degrees) of third instar *Drosophila melanogaster* larvae of varying BPA dosage conditions. The mean degree of angular movement for each condition were as follows: control (41.51 degrees), .44 mM (28.26 degrees), 4.4 mM (29.79 degrees), and 44 mM (53.29 degrees). Each condition exhibited variability, as shown graphically by the error bars on each column. The standard error values for each condition were as follows: control (5.85), .44 mM (5.3), 4.4 mM (3.73), and 44 mM (4.85). It is also important to note that, due to the differing number of angular turns for each condition, the data populations among each condition differed greatly in number, rendering each population greatly varied from one another. T-test values for each condition were calculated as follows: .44 mM (0.096), 4.4 mM (0.079), and 44 mM (0.134). None of the experimental conditions reached the statistical significance threshold of 0.05. To account for unequal variance, a Bonferroni correction was run, yielding a corrected significance threshold of 0.0167. None of the conditions reach this corrected significance threshold. From one-way ANOVA analysis, an F ratio was calculated as 6.5, and a P value was calculated as 0.0003.
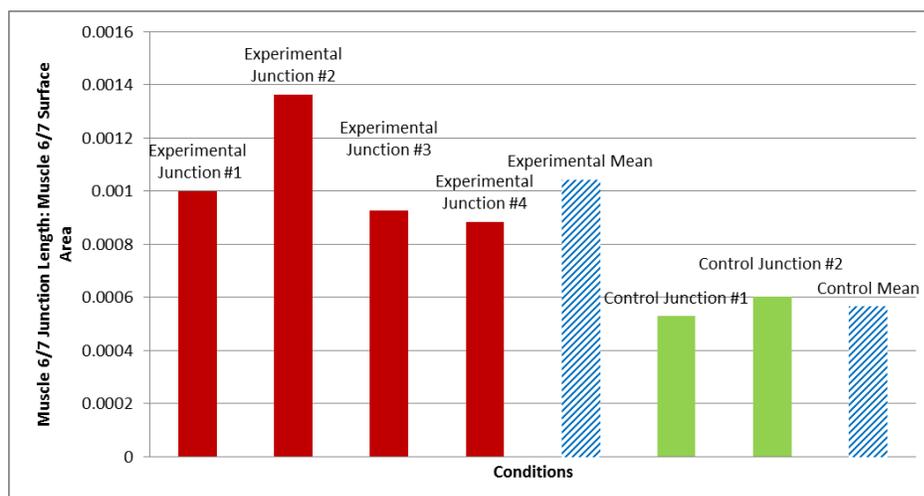

Figure 6: The ratio of neuromuscular junction length to muscle area of muscles 6 and 7. The experimental condition (blue) had an average length:surface area ratio of 9.94 X 10e-4 with a standard deviation of 2.19 X 10e-4 . The control condition (green) has a ratio of length:area ratio of 5.63 X 10e-4 and a standard deviation of 5.35 X 10e-5. The control ratio was 4.31 X 10e-4 units less than the experimental condition, which is a few standard deviations away from the mean.